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13. ABSTRACT (Maximum 200 words)

We have succeeded in establishing C. elegans as a small animal model of EPEC infection. With DARPA support we have demonstrated that EPEC bacteria kill nematodes faster than laboratory control strain MG1655 in the simple kill assay. As predicted, by fluorescent microscopy using bacteria labeled with green fluorescent protein (GFP), EPEC are found in greater numbers in the nematode gut compared to the control strain. EPEC bacteria colonize, and persist within the gut at least to forty-eight hours post-infection, and recent clinical isolates exhibited more robust colonization than the prototypical EPEC strain E2348/69. We found that neither the type III secretion system nor the type IV bundle-forming pilus (BFP) were necessary for colonization. Our most significant finding was that the global regulator Ler is necessary for EPEC to colonize the C. elegans gut. These findings resulted in a report to be published in the January 2006 issue of the ASM journal Infection and Immunity; the page proofs are included as an addendum. We envision the C. elegans model of infection to be useful in identifying potential chemotherapeutic agents against EPEC disease, as well as diseases caused by related gram-negative bacteria, and studying host-microbe interactions, namely attachment, colonization and persistence.

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Background

Our laboratory investigates the molecular pathogenesis of enteropathogenic *E. coli* (EPEC), a leading cause of infant diarrhea in developing countries (Nataro and Kaper, 1998). EPEC is thought to be underreported in developed countries, and is the prototype organism of a group of pathogenic bacteria that cause attaching and effacing (AE) intestinal lesions (Levine *et al*., 1978; Nataro and Kaper, 1998). AE lesions are characterized by disruption of the epithelial cytoskeleton and formation of pedestals that protrude out from the host cell surface to cup the bacterium (Moon *et al*., 1983). A variety of Gram-negative pathogens are capable of forming AE lesions, including enterohemorrhagic *E. coli* (EHEC) serotype O157:H7, which causes hemorrhagic colitis and hemolytic uremic syndrome (HUS) (Tzipori *et al*., 1986).

Formation of the AE lesions are mediated by a type III secretion system (for review see Hueck 1998), a syringe-like structure that connects the host cell cytoplasm with the bacterial cytoplasm. Through this channel, the bacterium secretes effector molecules, subverting host cell signaling events and thus causing disease. Type III secretion systems (TTSS) are found in many gram-negative pathogens of humans, other animals and plants. These include, among others, *Salmonella enterica*, an invasive diarrheal pathogen, *Yersinia pestis*, the agent causing bubonic plague, and *Chlamydia pneumoniae*, which has been implicated in heart disease (Schumacher *et al*. 2002). Because the histopathology caused by EPEC, genetic characterization, and phenotypic analysis of many virulence determinants, including the TTSS are well characterized in EPEC, it is an important model for the study of molecular bacterial pathogenesis in general.

In order to extend our knowledge of bacterial pathogenesis, understand its effects on host cells, and formulate effective vaccine or chemical therapies, the scientific community must have animal models of infection. Some animal models are currently available for certain pathogens, such as the infant mouse for *Salmonella enterica* or *Vibrio cholerae* infection, but animal colonies are costly to maintain, and in some institutions difficult to justify due to general public disapproval. Thus, there is a need for developing simple animal models for diverse groups of bacterial pathogens. This is certainly true for EPEC as no animal model is currently available to study its infection process.

Recent reports suggest that the nemotode *Caenorhabditis elegans* can be used as an infection model for diverse groups of bacterial pathogens, including those of both animals and plants (Couillalt and Ewbank, 2002; Tan *et al*. 1999). The ability to use *C. elegans* as a small animal model for EPEC infection would not only significantly contribute to our understanding of EPEC pathogenesis, but would also enhance our understanding of the mechanisms of infection of many Gram-negative pathogens. Potentially, these studies would lead to the development of novel, broadly applicable therapeutic agents and targets for vaccine therapy, not just for EPEC, but for other Gram-negative pathogens such as *E. coli* O157, *Shigella*, *Salmonella*, *Yersinia pestis*, and *Yersinia enterocolitica.* These therapies could potentially protect citizens as well as military personnel prior to deployment, and treat personnel in the field, minimizing outbreaks and maximizing military effectiveness. *C. elegans* is a well-studied organism,

used as a model for development and cell biology studies in more complex organisms, and is genetically tractable.

Research Approach

The first step in the project was to determine whether EPEC pathogens kill *C. elegans* in a simple kill assay on agar plates (Tan *et al*. 1999). Nematodes were propagated on nematode growth medium containing the food source *E. coli* strain OP50, synchronized using a bleaching procedure, and adult worms placed on separate agar plates containing the test EPEC pathogens and laboratory control strains. Synchronized, adult nematodes were incubated with these strains and were observed visually or by gently prodding with a sterile platinum wire to check viability over several days. We tested the prototype EPEC strain E2348/69, recent clinical isolates, attenuated pathogens, and laboratory control strains. We predicted that EPEC derivatives known to be attenuated in human disease would also be attenuated in their ability to kill *C. elegans*. For these initial assays our goal was to find the optimum culture conditions whereby the EPEC pathogens killed the nematodes more rapidly than laboratory control strains.

Qualitative and quantitative assays were performed to test our hypothesis that greater numbers of EPEC pathogens would be found in the *C. elegans* gut as compared to laboratory control strains. For the qualitative analysis, we transformed EPEC and laboratory control strains with a plasmid producing green fluorescent protein (GFP). We infected nematodes with these strains on agar plates using the identical assay conditions we developed for the kill assays. Infected nematodes were picked over several time points, exterior bacteria removed, and colonization of the gut by EPEC and control strains monitored by fluorescent microscopy. Simultaneous to these assays, we enumerated the bacteria inside the nematode gut by standard plate count assays. The nematodes were crushed using a sterile plastic pestle releasing rifampicin resistant bacteria, and then serial dilutions were plated on a selective medium and colony forming units (CFU) per nematode were determined.

Deliverables

Bacterial pathogens must accomplish four steps in order to cause disease: attach to a cell surface, colonize, or multiply to sufficient numbers, avoid elimination by the host immune system, and lastly damage the host. By disrupting colonization, diarrheal disease caused by EPEC and related pathogens can be prevented. Therefore, we envision using the *C. elgans* small animal model of infection to screen potential chemotherapeutic agents that prevent and/or disrupt EPEC colonization, and perhaps screening for therapies against a wide range of related pathogens. Screening of therapeutic agents can only be accomplished by using animal models, as human volunteers are of course inappropriate for this type of study.

The *C. elegans* model of EPEC infection will also be useful for the general study of host-pathogen interactions, and perhaps facilitate the identification of candidate vaccine

antigens. Human volunteer studies were used previously to identify the only two established EPEC virulence factors, the bundle-forming pilus (BFP) and intimin protein (Bieber *et al*. 1998; Schauer and Falkow, 1993). Both of these antigens are involved in attachment and colonization of host cells. Bacterial pathogenesis is multi-factorial; therefore, there is a great need for an infection model to identify additional EPEC virulence factors, and those contributing to disease in related pathogens. This need has been recognized by other researchers as well, and forms the basis of identifying potential vaccine antigens and chemotherapeutic agents.

In terms of BW targets, this work is applicable to the NIAID Category A Priority Pathogen *Yersinia pestis*. For the NIAID Category B Priority pathogens our work is applicable to the food and waterborne diarrheagenic *E. coli* strains, including the serotype O157:H7, pathogenic Vibrios, *Shigella*, *Salmonella*, and *Yersinia enterocolitica*. In terms of clinical targets, enterohemorrhagic *E. coli*, serotype O157:H7 is the most serious threat to public health in the US. However, Shigella and Salmonella spp. also cause significant morbidity and mortality annually.

The main deliverable of the project is an infection system to screen for potentially effective chemotherapeutic agents that inhibit and/or prevent colonization by the above pathogens. We will investigate the interactions of EPEC with the nematode gut epithelial cells, comparing results to the characterized interactions of EPEC with human epithelial cells, and strive to deliver a relevant, functional small animal model for screening chemotherapeutics by the end of the granting period, 8/31/2005.

Progress

Summary

We have succeeded in establishing *C. elegans* as a small animal model of EPEC infection. With DARPA support we have demonstrated that EPEC bacteria kill nematodes faster than laboratory control strain MG1655 in the simple kill assay. As predicted, by fluorescent microscopy using bacteria labeled with green fluorescent protein (GFP), EPEC are found in greater numbers in the nematode gut compared to the control strain. EPEC bacteria colonize, and persist within the gut at least to forty-eight hours post-infection, and recent clinical isolates exhibited more robust colonization than the prototypical EPEC strain E2348/69. We found that neither the type III secretion system nor the type IV bundle-forming pilus (BFP) were necessary for colonization. Our most significant finding was that the global regulator Ler is necessary for EPEC to colonize the *C. elegans* gut. These findings resulted in a report to be published in the January 2006 issue of the ASM journal *Infection and Immunity*; the page proofs are included as an addendum. We envision the *C. elegans* model of infection to be useful in identifying potential chemotherapeutic agents against EPEC disease, as well as diseases caused by related gram-negative bacteria, and studying host-microbe interactions, namely attachment, colonization and persistence.

Detailed progress for the quarter

The details of our findings can be found within the article titled, "The Global Regulator Ler Is Necessary for Entreopathothenic *Escherichia coli* Colonization of *Caenorhabditis elegans*" included as an addendum.

Future work

I have chosen to extend the *C. elegans* infection model to be used with ETEC pathogens for two reasons. First, there is a greater market for developing therapeutics designed to combat ETEC infections as opposed to EPEC because ETEC affects adult travelers and military personal in developing countries. Second, over twenty surface antigens, mostly fimbriae, have been described for ETEC, but it is unclear what role, if any, these structures play in the disease process.

We have demonstrated in the simple kill assay that ETEC bacteria kill *C. elegans* faster than laboratory control strains. Furthermore, the virulence plasmid-lacking strain H10407P appears attenuated for killing compared to the parent wild type ETEC strain H10407. The virulence plasmid encodes the CFA/I fimbrial antigen and the heat stable toxin, and thus further experimentation will be necessary to determine what role these virulence determinants play in the observed phenotype. As for EPEC, initial evidence demonstrates a slow-kill phenotype for ETEC, consistent with killing of nematodes by infection as opposed to toxin production. Thus our evidence is suggestive of a role for the CFA/I antigen, which is important in disease in humans (Wolf, 1997), in nematode killing, but this point must be addressed directly through further experimentation using genetically defined strains. An alternate explanation is that the CfaR regulator, also encoded on the virulence plasmid, partially controls ETEC killing of *C. elegans*.

Consistent with the simple kill assay results, we observed increased numbers of ETEC pathogens compared to the control strain MG1655 in the *C. elegans* gut by standard plate count and fluorescent microscopy assays. ETEC persists within the nematode gut at least forty-eight hours post-infection, and initial assays indicate that the H10407P strain lacking the CFA/I fimbria is slightly attenuated for colonization compared to strain H10407 containing this antigen. These promising initial studies indicate that it is most likely also feasible to use the *C. elegans* model of infection for the causative agent of traveler's diarrhea. We are therefore now screening anti-infective compounds to prevent and/or disrupt ETEC infection of the *C. elegans* gut. Once proof of concept has been established for the screening procedure, I plan to apply for a patent as this technology will be new, usable and not obvious from the existing literature.

In conclusion I see the nematode model as a system amenable to developing anti-*E. coli* pathogen therapies and perhaps more broadly anti-enteric pathogen therapies, and for acquiring a better understanding of the role of bacterial adhesins in infection. Future modifications to the system may include using nematodes that can be incubated at human host body temperature, such as *Panagrellus redivivus*, in order to properly express

bacterial virulence factors, and/or genetically modifying nematodes to express human specific intestinal epithelial cell receptors.

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